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Vasopressin immunoreactivity, but not vasoactive intestinal polypeptide, correlates with expression of circadian rhythmicity in the suprachiasmatic nucleus of voles ☆

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Abstract

In common voles (*Microtus arvalis*), natural variation in locomotor behavior can be exploited to study the mechanism of pacemaker control over circadian timing of behavior. Here we studied daily patterns in numbers of neuropeptide immunoreactive suprachiasmatic nucleus neurons in rhythmic, weakly rhythmic, and non-rhythmic voles. Circadian rhythmic voles showed circadian variation in numbers of vasoactive intestinal polypeptide and vasopressin immunoreactive suprachiasmatic nucleus neurons with a peak at zeitgeber time 0. In contrast, voles with weak or no circadian rhythmicity exhibited similar fluctuations for vasoactive intestinal polypeptide, but a continuous, non-rhythmic high profile for vasopressin. Vole suprachiasmatic nucleus neurons do not produce somatostatin or substance P. We conclude that the vasopressin system in the common vole suprachiasmatic nucleus acts as a principal correlate with expression of circadian behavior, in contrast to vasoactive intestinal polypeptide, somatostatin, and substance P. We also conclude that high levels of vasopressin immunoreactivity in the non-rhythmic vole suprachiasmatic nucleus is in line with previously demonstrated hampered release, probably resulting in vasopressin accumulation in the suprachiasmatic nucleus. Vasopressin could be a candidate in mediating output of the vole circadian clock, leading to circadian expression of locomotor behavior.

Keywords: Suprachiasmatic nucleus; Vasopressin; Locomotor behavior; Neuropeptides; Common vole (*Microtus arvalis*); Stereology

1. Introduction

The hypothalamic suprachiasmatic nucleus (SCN) is the locus of the pacemaker for circadian behavioral rhythms (Ralph et al., 1990; Silver et al., 1996). The SCN produces a diversity of neuropeptides (Van den Pol and Tsujimoto, 1985; Inouye and Shibata, 1994), but how and which signals from the SCN lead to circadian behavior is still unanswered. In this respect arginine-vasopressin (AVP) has received much attention. Circadian rhythmicity has been observed in AVP content of the SCN using micropunches (Södersten et al., 1985; Tominaga et al., 1992), and immunostaining in situ (Okamura, 1996). Evidence exists that AVP is involved in regulating timing programs of locomotor behavior, most notably in the common vole, *Microtus arvalis* (Gerkema et al., 1994), but also in mice and rat

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(Bult et al., 1993; Van der Zee et al., 2005; Wollnik and Bihler, 1996). Non-rhythmic voles exhibited significantly higher numbers of AVP-immunoreactive (AVPir) neurons in the SCN at zeitgeber time (ZT) 3 than rhythmic voles do (Gerkema et al., 1994). This difference was retained in long-term organotypic SCN cultures (Jansen et al., 1999). Furthermore, non-rhythmic voles have low levels of AVP release with no circadian fluctuation, in contrast to rhythmic voles (Jansen et al., 2000).

In the rat SCN, the presence of vasoactive intestinal polypeptide (VIP), somatostatin, and substance P have been described (Van den Pol and Tsujimoto, 1985; Okamura et al., 1987), and interaction of such peptides with each other and AVP might contribute to the functioning of the SCN and its output systems (Albers et al., 1991; Shinohara et al., 1995; Watanabe et al., 2000; Jansen et al., 2003; Van der Zee et al., 2005). Somatostatin neurons have a high degree of connectivity with other SCN neurons (Biemans et al., 2002). Besides somatostatin, substance P is also able to reset the phase of the clock (Liu et al., 2000), while substance P and somatostatin are found to colocalize in the SCN and their fibers synapse on VIP and AVP neurons in the rat SCN (Daikoku et al., 1992). Several mechanisms have been proposed for the regulation of VIP and AVP by neuropeptides like somatostatin and substance P in the mammalian SCN.

We investigated whether VIP, somatostatin, and substance P are expressed in the vole SCN and, if so, we determined the numbers of such immunoreactive neurons in behaviorally rhythmic, weakly rhythmic and non-rhythmic voles throughout the circadian cycle. The AVP-behavior correlation in the vole SCN has been shown for only one circadian time point and leaves us with the question whether we can extrapolate this result to the full circadian cycle. We therefore also examined whether the phenomenon of high numbers of AVPir neurons in the SCN (Jansen et al., 2000) of non-rhythmic (and to a lesser extent in weakly rhythmic voles) is present throughout the circadian cycle, and whether a circadian fluctuation exists in numbers of AVPir SCN neurons in rhythmic voles.

2. Materials and methods

2.1. Animals

In total, 96 adult male common voles (*M. arvalis*, 2 months of age, 20 ± 5 g) were used from our colony maintained in Haren which is based on individuals trapped in the Lauwersmeer (53°20′N:6°16′E). Animals were individually housed in transparent cages ($25 \times 25 \times 30$ cm) equipped with a running wheel and connected with a transparent nest box ($17 \times 11 \times 13$ cm) provided with wood shavings. Food (Hope farms® mouse pellets, Woerden, The Netherlands) and tap water were available *ad libitum*. Temperature (20 ± 1°C) and humidity (70%) were kept constant during the experiment. The Animal Experimental Committee of the University of Groningen approved the experiment (DEC No. 2091).

Rhythmic voles ($n = 6$) were killed at ZT 0 for the investigation of the presence of VIP, somatostatin, and substance P (the presence of AVP in the vole SCN has been shown extensively; Gerkema et al., 1994; Jansen et al., 1998, 1999, 2000; Van der Zee et al., 1999). Eighty-four voles were used for circadian profiling, followed by immunoprocessing as described below. Per time point, 21 voles were used and assigned to the three behavioral categories leaving $n = 7$ voles for each individual point. Finally, 12 additional rhythmic voles were either killed at ZT 0 ($n = 6$) or ZT 12 ($n = 6$) for the estimation of the total number of SCN neurons by cresyl violet staining.

2.2. Immunocytochemistry (ICC)

Voles used for this study were killed under deep anesthesia with 0.75 ml of 0.2% sodium pentobarbital injection. Brains were quickly removed from the skull and subjected to immersion fixation in 4% paraformaldehyde in 0.1 M phosphate-buffer for 12 h. Brains were rinsed with 0.01 M phosphate-buffered saline (PBS) and cryo-protected overnight in 30% buffered sucrose at 4 °C. Frontal sections (25 μm) were cut with a cryostat at the level of the hypothalamus. Free floating sections were rinsed with PBS and pre-incubated with normal goat serum (5%) and then incubated with a rabbit polyclonal IgG antibody anti-AVP (Truus'86, Netherlands Institute for Brain Research, Amsterdam, 1:5000), a rabbit polyclonal IgG antibody anti-VIP (Sigma, CA, USA), a rabbit polyclonal IgG antibody anti-somatostatin (a generous gift by Dr. C. Rougeot, Paris, France), or a rabbit polyclonal IgG antibody anti-substance P (Biotrend, Germany, 1:2000) overnight at 4 °C. After rinsing, all sections were subjected to the same protocol. Sections were exposed to biotinylated goat anti-rabbit IgG (F(ab′)2 fraction (Zymed, CA, 1:200)) for 2 h at room temperature. Subsequently, the sections were rinsed again in PBS and incubated with Streptavidin-Horseradishperoxidase (Zymed, CA, 1:200) for 1 h at room temperature. Triton X-100 (0.5%) was added to all incubation steps. The sections were thoroughly rinsed in PBS and Tris buffer (0.05 M, pH 7.4) and the protein–antibody complex was visualized with diaminobenzidine (30 mg in 100 ml Tris buffer, pH 7.4) as chromogen and 0.01% H_2O_2 for initiation of the staining reaction. Finally, sections were rinsed in PBS, mounted, air dried, and coverslipped for light microscopy and stereology. Brain tissue was processed for cresyl-violet staining with the same protocol as for ICC until the incubation step of the first antibody.

Instead, sections were mounted, stained with cresyl-violet and coverslipped.

2.3. Behavioral characterization

Voles were characterized as described before (Jansen et al., 2003). Briefly, 80 voles used for the circadian profiles of AVP and VIP were exposed to a 12:12 h light:-dark (L/D) cycle (lights on at 6:00 h (ZT 0): 350 ± 1 lux) for two weeks. After this, the animals stayed four weeks in constant low light (L/L) conditions (±2 lux). Subsequently, they were reexposed to a fortnight L/D treatment (12:12 h: lights on 6:00 h: ±350 lux) in order to re-entrain their activity patterns. Voles were assigned to three behavioral categories: rhythmic, weakly rhythmic and non-rhythmic, on the basis of visual inspection of their individual wheel running activity pattern. Within each of the three behavioral categories, animals were randomly assigned to four subgroups: ZT 0, 6, 12, and 18. Wheel running activity was recorded via a microswitch connected with a PC-based event recording system (ERS) and activity patterns were further analyzed, in order to check whether visual categorization was consistent with quantitative analyses. Wheel running records of the last 10 days under dim light conditions were analyzed as follows. We used Chi-square (χ^2) periodogram analysis (Sokolove and Bushel, 1978) as an index for rhythmicity. We calculated the difference Delta Q_p (ΔQ_p) between the significance line at $p = 0.05$ and the actual rhythmicity Q_p for all periods (Gerkema et al., 1994). Precision of rhythmicity, that is interdaily variation of the circadian phase markers, was determined by analysis of the coefficient of variation both for visually determined onsets of activity, and for cyclic centers of gravity, indicating the highest intensity of activity (Kenagy, 1980), in daily activity records. We calculated the location of center of gravity in a 24 h window, sliding with a 2-min step size, during 10 days. All calculated locations of center of gravity of activity referring to the same activity bout were averaged. Locations were defined to belong to a next (circadian) episode when they were located more than 16 h away from the previous one. Daily activity distribution over four 6 h blocks was analyzed after Van der Zee et al. (1999) with a slight modification: uniform distribution patterns were estimated by non-parametric one-way analysis of variance (Kruskal–Wallis), followed by post hoc χ^2 tests for uniform distribution.

2.4. ICC quantification

Analysis of immunoreactive neurons was restricted to AVP and VIP immunoreactive neurons in the vole SCN, since no somatostatin and substance P immunoreactivity was found (see Section 3). Total numbers of AVPir and VIPir neurons were estimated using the optical fractionator method described by West (1993) and Madeira et al. (1997). Cell counting was carried out using a transparent disector probe which was placed in the ocular of an Olympus CH40 light microscope. Cells were counted at 1000× magnification. The fields of view were systematically sampled using a step size of 0.054 mm along the x-axis and 0.054 mm along the y-axis, and the disector counting frame was 729 μm^2. Hence, the area sampling fraction was 0.25. The section sampling fraction was 0.25, since one quarter of the sections was used for AVP immunocytochemistry and one quarter was used for VIP-immunocytochemistry. The tissue (thickness) sampling fraction, indicating the extension of the middle portion of the 25 μm slices which was used for analysis, was fixed at 0.62. Neurons were counted only when cytoplasmatic stainings were unambiguously apparent, and both hemispheres were sampled alternately. The minimum number of AVPir and VIPir neurons counted in one nucleus was 32 and 48, respectively. The numbers per nucleus were pooled (resulting in value Q) for each animal and total numbers (N) were calculated according to the formula ($N = 1/0.25 \times 1/0.25 \times 1/0.62 \times \sum Q$). Significant differences (two-way Friedman ANOVA) in numbers of immunoreactive neurons within behavioral categories were interpreted as circadian variation. Post hoc Tukey tests were used to compare average numbers of immunostained neurons at different ZTs. For the estimation of the total number of neurons in cresyl-violet stained sections, the same stereological protocol was followed, except that the disector frame was 808 μm^2.

3. Results

3.1. Behavioral data

Visual assignment of individual records to the categories circadian rhythmic, weakly rhythmic and non-rhythmic was tested by four independent analytical tools (Table 1). Analysis of daily activity distribution patterns showed a significant difference in the occurrence of uniform distribution of daily activity between three behavioral categories ($\chi^2 = 49.62$; $p < 0.001$). Similar results were obtained in the comparison of uniform distributions in rhythmic and non-rhythmic voles, and between rhythmic and weakly rhythmic voles, respectively. Only weakly rhythmic voles and non-rhythmic voles did not differ significantly. Visual distinction of individual records between animals with strong, weakly or absent circadian patterns in their actograms was corroborated by a significant difference in peak ΔQ_p values in the 22–26 h range of the χ^2-periodogram between the three behavioral categories. Although at a lower significance level, also a difference was found between peak ΔQ_p

Table 1
Analysis of wheel running records under constant dim light conditions

Visual score	Rhythmic (R)	Weakly rhythmic (W)	Non-rhythmic (N)	Overall comparison	Comparison R–W	Comparison R–N	Comparison W–N
Uniform distribution	$n = 0$	$n = 2$	$n = 20$	$p < 0.0001$[a]	N.S.[a]	$p < 0.001$[a]	$p < 0.0005$[a]
ΔQ_p value	1321 ± 63	406 ± 48	264 ± 49	$p < 0.005$[b]	$p < 0.001$[c]	$p < 0.001$[c]	N.S.[c]
Cov. Grav.	0.048 ± 0.021	0.112 ± 0.017	0.161 ± 0.024	$p < 0.001$[b]	$p < 0.001$[c]	$p < 0.05$[c]	$p < 0.05$[c]
Cov. Onset	0.117 ± 0.024	0.302 ± 0.059	1.240 ± 0.042	$p < 0.001$[b]	$p < 0.001$[c]	$p < 0.001$[c]	N.S.[c]

Voles were categorized according to actogram patterns in rhythmic, weakly rhythmic and non-rhythmic (see also Jansen et al., 2003).
N.S. non-significant.
ΔQ_p, rhythmicity index obtained by χ^2 periodogram analysis (mean ± SEM); Cov. Grav., coefficient of variation of center of gravity (mean ± SEM); Cov. Onset, coefficient of variation of activity onset (mean ± SEM). Sample size = 31, 25, and 24 for rhythmic, weakly rhythmic and non-rhythmic voles, respectively.
[a] χ^2 test.
[b] Kruskal–Wallis one-way ANOVA.
[c] Mann–Whitney U test.

values of weakly rhythmic and non-rhythmic voles ($p < 0.005$; Mann–Whitney U test, two tailed; Table 1).

Precision of circadian pattern, based on the daily points of gravity, differed between the three behavioral categories (Kruskal–Wallis, one-way ANOVA, $p < 0.001$). Comparisons of the precision of the activity rhythm based on determination of the coefficient of variation of activity onset revealed a significant difference between the three behavioral categories (Kruskal–Wallis, one-way ANOVA, $p < 0.001$). It should be noted that differences between weakly rhythmic and non-rhythmic voles were not as strong, indicated by indistinguishable distribution over 24 h episodes and precision based on activity onset (Table 1). In Fig. 7, wheel running actograms of the three behavioral categories (and tau's) are shown.

3.2. Immunoreactivity

A plexus of AVPir fibers and nerve terminals was observed in the dorsomedial vole SCN, at all ZTs and in all three behavioral categories (Fig. 1). Some scattered AVPir terminals and neurons were found in the ventral SCN, but most AVPir neurons were found in the dorsomedial SCN. Total numbers of AVPir SCN neurons varied significantly with respect to behavioral categories and ZT (Fig. 2; Friedman, two-way ANOVA, $F(2.34) = 6.913$; $p < 0.005$). A significant fluctuation in numbers of AVPir SCN neurons was found in behaviorally rhythmic voles over time of day (Tukey post hoc test, $F(3.21) = 3.956$; $p < 0.05$; Fig. 2), but neither in weakly rhythmic nor in non-rhythmic voles (Tukey post hoc test, $F(3.21) = 2.72$ and 2.35 for weakly and non-rhythmic voles, respectively; $p > 0.05$). Although numbers of AVPir SCN neurons of weakly rhythmic and non-rhythmic voles did not differ significantly from each other at any ZT (Tukey post hoc test; $F(3.28) = 0.56$, 0.22, 1.90, and 1.08 for ZT 0, 6, 12, and 18, respectively; $p > 0.05$), it should be noted that at ZT 12 and 18 weakly rhythmic voles had intermediate numbers of AVPir SCN cells. Numbers of AVPir neurons in rhythmic voles differed significantly from those in non-rhythmic and weakly rhythmic voles at ZT 6, 12, and 18 (Tukey post hoc test; $F(3.28) = p < 0.05$), but not at ZT 0, when rhythmic voles showed a peak in number of AVPir neurons (Figs. 1 and 2).

Numerous VIPir neurons were observed in the ventral region of the SCN throughout the rostro-caudal axis (Fig. 3). Many VIP immunopositive boutons and fibers were present throughout the ventral part, extending to the dorsomedial part of the SCN. VIPir fibers originating from the ventral SCN area had many nerve terminals in the dorsal part of the SCN (Fig. 3). Variability in VIP, however, was particularly reflected in numbers of VIPir neurons between different ZTs. Irrespective of ZT or behavioral category, in rhythmic, weakly rhythmic, and non-rhythmic animals a significant circadian pattern was present in the SCN (Fig. 4; Friedman, two-way ANOVA, $F(2.34) = 5.844$; $p < 0.005$). Subsequent Tukey post hoc analysis revealed that the three behavioral categories did not differ significantly from each other in the number of VIPir neurons ($F(1.87) = 0.34$; 0.13; 0.45; 0.09; $p > 0.05$ for ZT 0, 6, 12, 18, respectively) (Fig. 4). Separately, all three behavioral categories showed circadian fluctuation (Tukey post hoc test; $F(2.36) = 5.78$; 7.19; 4.72; $p < 0.05$ for rhythmic, weakly rhythmic, and non-rhythmic, respectively).

Within the SCN of common voles no (specific) immunoreactivity for somatostatin and substance P could be detected (Fig. 5). Immunoreactive cells for somatostatin and substance P were found just outside the SCN, and in other hypothalamic areas like the peri- and paraventricular nucleus (Fig. 6).

To test whether a fluctuation in total number of neurons contributes to the observed differences in numbers of AVPir- and VIPir neurons, cresyl violet stainings were performed on sections from two groups of rhythmic voles that were killed at ZT 0 or ZT 12. The average number of neurons at ZT 0 was 12,348 (SEM = 1556;

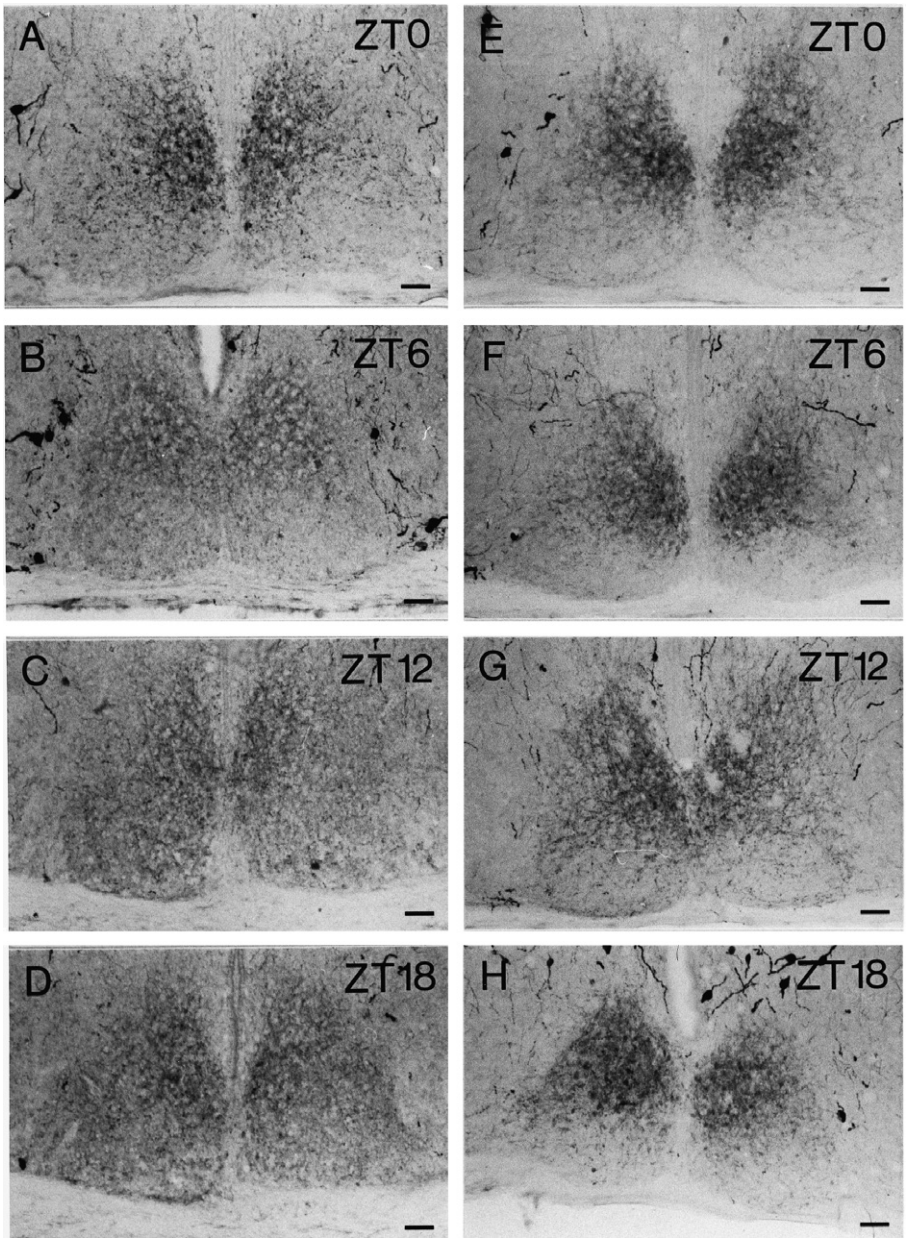

Fig. 1. Representative photomicrographs of AVP immunoreactivity in the SCN of rhythmic voles at ZT 0 (A), ZT 6 (B), ZT 12 (C), and ZT 18 (D), and of non-rhythmic voles at ZT 0 (E), ZT 6 (F), ZT 12 (G), and ZT 18 (H). Note the AVPir neurons just outside the SCN of the rhythmic vole at ZT 6, indicating that low numbers of AVPir neurons in the SCN are not a flaw in immunostaining. Scale bars = 50 μm.

$n = 6$), and 13,762 (SEM = 1923; $n = 6$) at ZT 12 (Not significant, Mann–Whitney U test, $p > 0.05$).

4. Discussion

4.1. Circadian fluctuation of neuropeptide expression in rhythmic voles

Behaviorally rhythmic voles express circadian fluctuation in AVPir and VIPir cell numbers in the SCN. The peaks and troughs in the observed circadian rhythms for these peptides are in line with the literature on levels of AVP and VIP content in the rat SCN under L/D cycle (Takahashi et al., 1989; Shinohara et al., 1993; Okamura, 1996), as well as AVP and VIP release in vivo and *in vitro* (Shinohara et al., 1995). The current data are also in line with the AVP output correlate protein kinase Cα (Jansen et al., 2003).

The circadian fluctuation in number of peptidergic neurons is best explained by a decrease in content due to release across the light period, followed by an increase as a consequence of protein synthesis, as also suggested by others for a variety of SCN neuropeptides

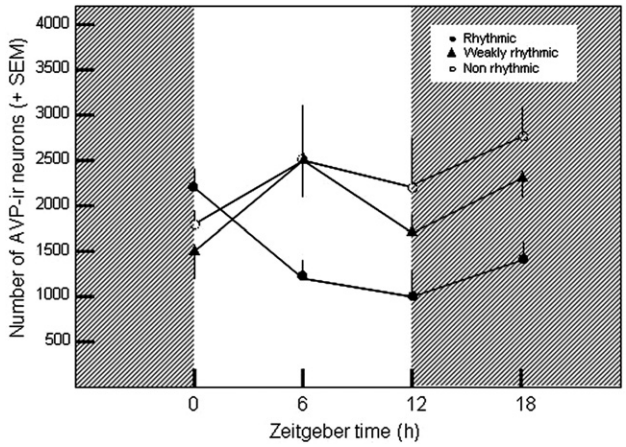

Fig. 2. Average numbers of AVPir neurons in the SCN of rhythmic, weakly rhythmic and non-rhythmic voles. Bars indicate standard error of the mean. Asterisks indicate the significant difference in number of AVPir neurons between the non-rhythmic voles and rhythmic voles at ZT 6, ZT 12, and ZT 18. Grey areas represent the dark period.

(Shinohara et al., 1993). We ruled out the possibility that the numerical differences in number of peptidergic SCN neurons was caused by changes in total cell numbers in the SCN. While a maximum change in number of peptidergic neurons was observed between ZT 0 and 12, counts of cresyl-violet stained cells did not differ significantly between these ZTs. Voles do not express somatostatin or substance P in the SCN but do show immunoreactivity in various other hypothalamic nuclei. This absence in the SCN is unlikely to be due to unrecognized epitopes of the somatostatin or substance P molecule by the used antibodies or because the animals were not pretreated with colchicine (which enhances peptide concentrations in the cell bodies), since just outside the SCN and in the paraventricular area immunoreactive fibers and scattered neurons containing somatostatin and substance P were frequently observed. Moreover, also no substance P fibers and terminals were encountered in the SCN, of which the detection is independent of colchicine treatment (Larsen, 1992).

Somatostatin shows endogenous circadian rhythms and could be an important modulator of local circuit interactions in the SCN (Shinohara et al., 1991; Fukuhara et al., 1993; Van Esseveldt et al., 2000; Biemans et al., 2002). In contrast to the solely intrinsic origin and projections of somatostatin within the SCN (Card et al., 1988), substance P originates additionally from fibers arising from the retina (Mikkelsen and Larsen, 1993). The latter substance P is involved in conveying light information (Abe et al., 1996), whereas the function of intrinsic substance P is less clear. Interestingly, both substance P and somatostatin are localized in the intermediate zone of the SCN and these neuropeptides are largely co-expressed in the same neurons (Larsen, 1992; Mikkelsen and Larsen, 1993; Tanaka et al., 1996). Absence of substance P and somatostatin neurons in the vole SCN may indicate a lack of expression in otherwise present cells, or the absence of this class of neurons. If the latter is true, it might also indicate that the vole SCN has in general a poorly developed or even absent intermediate zone. Notwithstanding the known species-variability in the presence of substance P in the SCN (Mick et al., 1992, and references therein), it is clear that the neuropeptidergic neurochemistry of the vole SCN differs from that of other rodents, e.g. rat, mouse and hamster (Van den Pol and Tsujimoto, 1985; for review see Van Esseveldt et al., 2000; Van der Veen et al., 2005).

4.2. Neuropeptide expression and correlation with timing of locomotor behavior

First, it should be stressed that the strength in the circadian organization of locomotor behavior in voles was assessed in constant dim light conditions, whereas the neuropeptidergic analyses were done after all animals were re-entrained to a 12:12 LD cycle. We followed this standard procedure for three reasons: (1) neuropeptide concentrations are influenced by constant light conditions (Shinohara et al., 1993; Gerkema et al., 1994), (2) no circadian phase can be determined in weakly and non-rhythmic voles, and (3) we are primarily interested in elucidating basic malfunctions of the SCN of non-rhythmic voles under natural lighting conditions.

A striking difference has been found in the expression of AVP in SCN neurons between behaviorally rhythmic and weakly/non-rhythmic voles, but not for VIP. VIP rhythms in voles may be solely dependent on the presence of an LD cycle, unrelated to the strength of the underlying circadian clock system. Such a difference in AVP immunoreactivity in the SCN in relation to locomotor behavior was originally studied at one time point (ca. ZT 3; Gerkema et al., 1994). These differences have led to the idea of an aberration in AVP release, and the hypothesis that AVP is accumulated and confined within SCN neurons (the AVP release deficit hypothesis). This hypothesis has recently been confirmed; hampered AVP release in the SCN of non-rhythmic voles and hence accumulation of AVP was shown using long-term organotypic SCN cultures (Jansen et al., 2000). Besides severely reduced AVP release, also a lack of circadian fluctuation in this release was found in non-rhythmic voles. Here, examination of AVPir SCN neurons was extended over the full zeitgeber cycle. These data also point to an absence of circadian fluctuation in AVP content in the SCN of non-rhythmic voles. The results of this study are therefore fully in agreement with hampered AVP release in non-rhythmic voles.

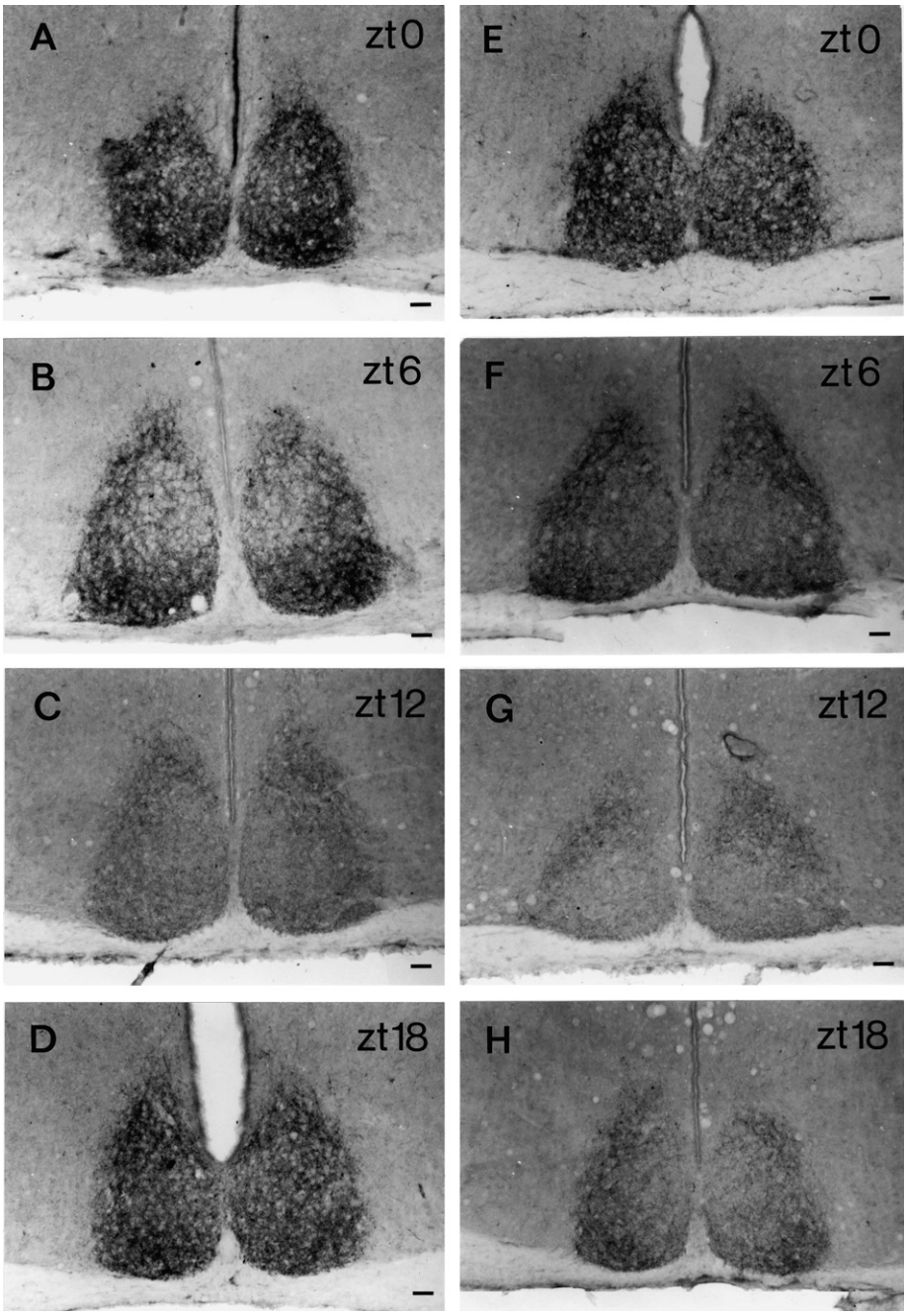

Fig. 3. Representative photomicrographs of VIP immunoreactivity in the SCN of rhythmic voles at ZT 0 (A), ZT 6 (B), ZT 12 (C), and ZT 18 (D), and of non-rhythmic voles at ZT 0 (E), ZT 6 (F), ZT 12 (G), and ZT 18 (H). Scale bars = 50 μm.

4.3. Putative functions and interactions of SCN neuropeptides in the vole

The data of weakly and non-rhythmic voles clearly suggest that the presence of a VIP rhythm is not sufficient to implicate the presence of AVP rhythms in the vole SCN. Shinohara et al. (1995) demonstrated the persistence of circadian release patterns of VIP, coupled to that of AVP depending on astrocyte conditions. With antimitotic treatment, reducing the number of glia cells in organotypic cultures, rhythms of VIP and AVP release showed different periods, in long-term organotypic rat SCN cultures. However, no circadian patterns in VIP release were found *in vitro* in organotypic SCN cultures of vole pups, with unknown state of circadian rhythmicity. Without antimitotic treatment, AVP release occurred in a circadian fashion in part of these same cultures (Gerkema et al., 1999). Apparently, the coupling between AVP and VIP rhythms *in vitro* is less pronounced in voles compared to rats.

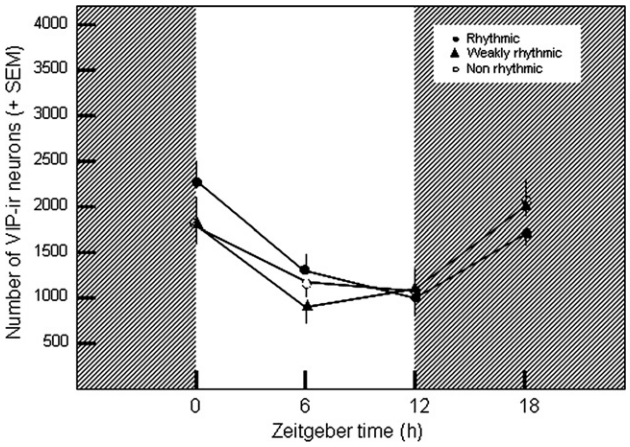

Fig. 4. Average numbers of VIPir neurons in the SCN of rhythmic, weakly rhythmic and non-rhythmic voles. Bars indicate standard error of the mean. Grey areas represent the dark period.

It is believed that VIP in the SCN of rats codes for photic information and plays a crucial role in the induction of phase shifts (Shinohara et al., 1993; Shinohara and Inouye, 1995; Watanabe et al., 2000). Our data are in line with the idea that VIP in the SCN may be primarily involved in conveying light input rather than participating in the output pathways of the vole SCN such as AVP is.

AVP is the only neurochemical transmitter in the vole SCN that we could correlate with differential expression of behavior so far. In interaction with AVP, somatostatin and substance P might contribute to the circadian expression and rhythm amplification within the SCN of other rodents (Van den Pol and Tsujimoto, 1985; Ingram et al., 1998). The lack of somatostatin and substance P expression in the SCN may render the neuropeptide contribution of the circadian pacemaker coupling with behavior more dependent on VIP and/or AVP. Since only AVP correlates with behavior, it is tempting to speculate that AVP could be critical in the coupling of pacemaker activity and the timing of circadian organization of vole behavior.

It remains to be investigated whether the AVP release deficit of non-rhythmic voles is the causal factor of non-rhythmic locomotor behavior. Even so, additional studies are needed to reveal what physiological mechanism in this AVP system is disturbed or downregulated. A deviation in release mechanism that impairs AVP release could also disturb other neurochemical output of the

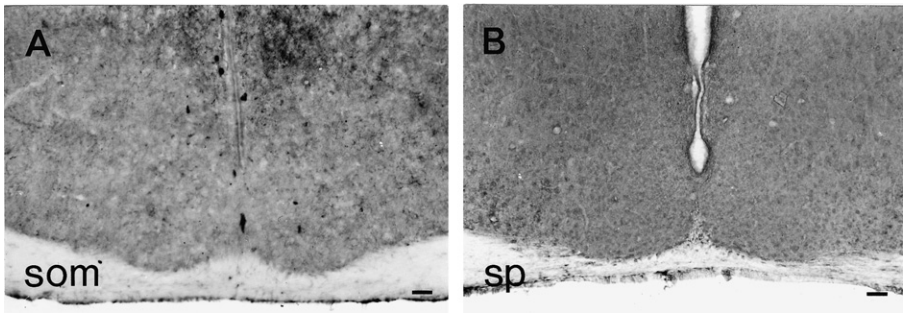

Fig. 5. Representative photomicrographs of the SCN of a rhythmic vole sacrificed at ZT 0, where sections were subjected to somatostatin (A) or substance P (B) immunostaining. No somatostatin-immunoreactive cells are observed in the vole SCN, while most somatostatin-immunoreactivity is present outside the SCN (A). Scale bars = 50 μm.

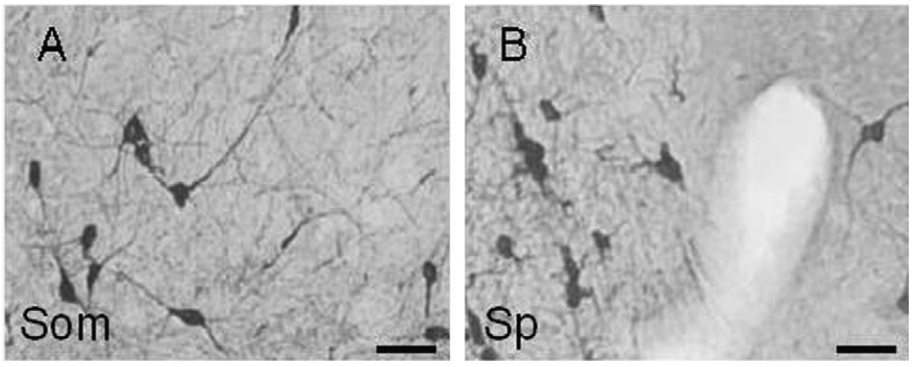

Fig. 6. Photomicrographs of the paraventricular nucleus of a rhythmic vole sacrificed at ZT 0, where sections were subjected to somatostatin (A) or substance P (B) immunostaining. Scale bars = 50 μm.

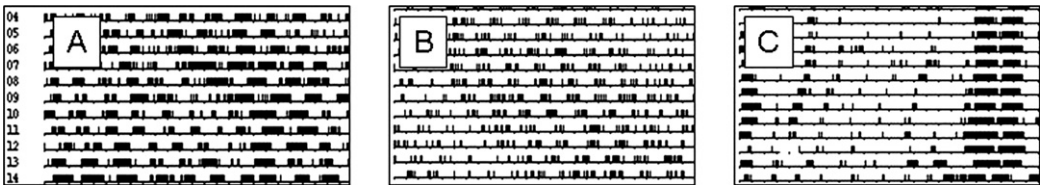

Fig. 7. Actograms (A–C) of wheel running activity of a non-rhythmic (A), a weakly rhythmic (B), and a rhythmic vole (C) during continuous low light (±2 lux). Average tau for weakly rhythmic voles is 24.40 h and for rhythmic voles 24.25 h.

SCN (e.g. PKCα, GABA) (Jansen et al., 2003; Kalsbeek et al., 2006), which could also be critically involved in the organization of vole circadian behavioral patterns.

Conflict of interest statement

None declared.

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